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14. ABSTRACT

Parkinson's disease results in part from the loss of dopamine neurons. We hypothesize that exercise reduces the vulnerability of dopamine neurons to neurotoxin exposure, which is modulated by stress. We have outlined experiments to test this hypothesis in rats treated with one of several neurotoxins, beginning with 6-hydroxydopamine. Over the past year, we increased the size and training of our research team and made a number of observations of direct relevance to our hypothesis. We also have received permission to expand our original Statement of Work to include critical studies on the mechanism of the actions of exercise, using both in vivo and in vitro approaches. Our focus continues to be on the effects of stress and exercise on the vulnerability of DA neurons, and the role played in these phenomena by trophic factors and intracellular signaling cascades.

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Basal ganglia, dopamine, ERK, exercise, MAPK, neuroprotection, Parkinson's disease, preconditioning, LRRK2

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Introduction:

Parkinson's disease (PD) results in part from the progressive loss of dopamine (DA) neurons projecting from substantia nigra (SN) to striatum. Although the cause of this neurodegenerative process is unknown, one candidate is oxidative stress. This is likely to result from exposure to environmental toxins, perhaps coupled with one or more bases for increased vulnerability. Such increased vulnerability could include genetic predisposition, emotional or physical stress, or exposure to certain recreational drugs. Our hypotheses regarding these matters are under continual refinement as we and others collect and publish additional data. Currently, we are guided by the following conceptualization (see also Figure 1 and accompanying legend):

> *Exercise reduces the neurological consequences that would otherwise develop in some individuals as a result of toxin exposure. This results in part from an exercise-induced increase in neurotrophic factors (NTFs) acting thought intracellular cascades, such as those involving the kinases ERK and PI3K. This NTF/kinase axis produces its ameliorating effects in several complimentary ways, including (a) reducing the vulnerability of DA neurons to the toxin, (b) increasing the functional capacity of residual DA neurons, and (c) facilitating the transfer of some functions previous attributable to DA neurons to other projections, including those utilizing serotonin. These effects of exercise are promoted by low levels of stress (which exercise itself may elicit) but are inhibited by high levels of stress.*

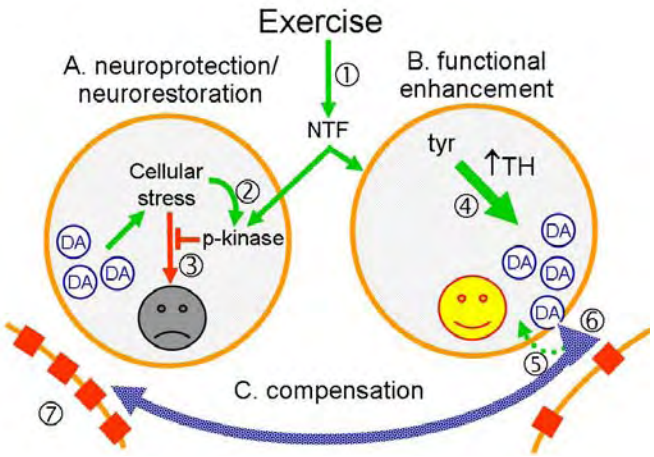

Figure 1: A schema for the impact of exercise on DA neurons. Cellular stress can result from many factors including ROS, mitochondrial deficiency, and proteasomal dysfunction. These insults can, in turn, be initiated as a result of environmental or genetic factors and be influenced by variables such as diet and physical activity. We hypothesize that (1) Exercise increases expression of several neurotrophic factors (NTFs), including GDNF and BDNF. These, in turn (2) activate intracellular signaling cascades including Ras/ERK and PI3K/Akt, leading to the phosphorylation of protein kinases (p-kinase) and activation of downstream elements. (3) Mild cellular stress can lead to a similar activation of survival cascades, which we and others have termed *preconditioning*. Together, these actions can often **(A) *protect DA neurons from death.*** NTFs can also **(B) *increase the capacity of remaining DA neurons*** by (4) both activating and inducing TH, thereby increasing DA synthesis. This, coupled with (5) a decrease in DAT activity, leads to (6) increase DA overflow. Moreover, if the loss of DA neurons is large (typically >90%), there will be an (7) increase in DA receptors. Together with the increased DA overflow, this is to **(C) *compensate for DA neuron loss***. Some elements of our model are not shown, including a transfer of some functions to other systems (e.g., serotonin) and an alteration in synaptic number.

Below we summarize our studies performed in 2006-7 that are designed to test aspects of these hypotheses, and then briefly comment on our plans for the final year of our support.

Body:

1. GDNF can protect against 6-OHDA; however, the full effect is delayed.

Distribution of exogenous GDNF after intrastriatal administration: No GDNF immunoreactivity was observed in animals treated with 6-OHDA alone or with the 6-OHDA vehicle at any time point examined. Two weeks after infusion of GDNF, a large spread of GDNF immunoreactivity beyond the needle track was observed in the striatum of animals given GDNF, either alone or together with 6-OHDA. However, by 4 and 8 wks, GDNF was largely confined to the needle track. No GDNF was observed in the SN of any animals at any time point.

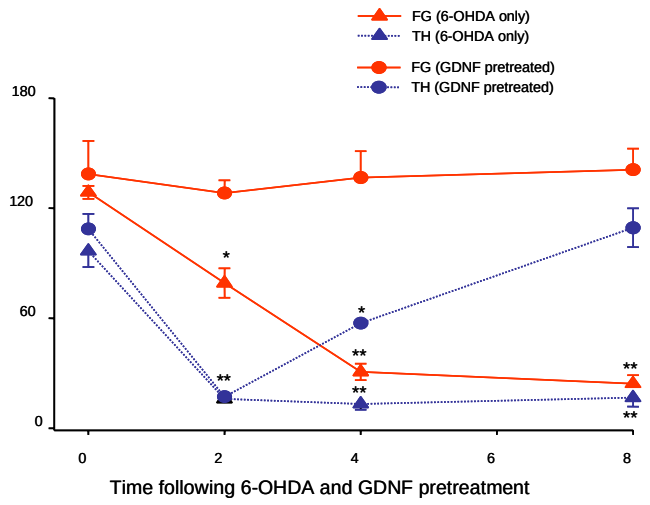

Figure 2 Effects of GDNF on TH^+ terminals in the striatum after 6-OHDA. Loss of TH was prevented by GDNF prior to 6-OHDA infusion into the striatum at 4 and 8 but not 2wk post-6-OHDA. No GDNF and GDNF+6-OHDA (2wk) animals displayed significant loss of TH when compared to sham animals (* $p<0.01$). This loss was prevented in GDNF+6-OHDA (4 wk) and GDNF+6-OHDA (8 wk) animals. All values are expressed as average lesion area in mm^2 ± SEM. Comparable data were obtained for DAT and VMAT2. Mean ± SEM.

Effect of GDNF on the 6-OHDA-induced loss of TH, DAT, and VMAT2 immunoreactivity in the striatum: At 2 wk post-operative, animals treated with 6-OHDA alone displayed a significant loss of each of the major phenotypic markers for DA neurons as assessed by an immunohistochemistry (IHC) analysis of striatum. These markers included tyrosine hydroxylase (TH), the high affinity DA transporter (DAT), and the vesicular monoamine transporter, type 2 (VMAT2). The loss remained constant for the 8-week duration of the study ($p < 0.001$). Pretreatment with GDNF 6 hrs prior to 6-OHDA infusion did not protect against the loss of these markers at 2 wks. However, these markers gradually recovered over 4-8 wks, with a ~70% reduction in lesion size at 4 wks and an ~80% reduction in lesion size at 8 wks ($p < 0.001$) (Figure 2). Treatment with vehicle or GDNF alone had no effect on levels of any of these phenotypic markers.

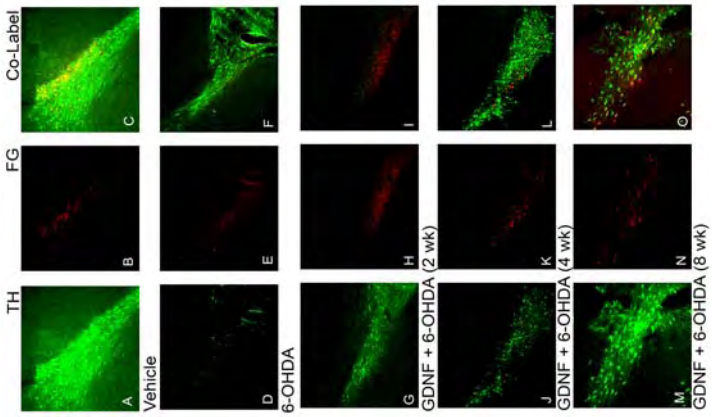

Figure 3: Effects of GDNF on loss of TH^+ and FG^+ cells in the SN after 6-OHDA. 6-OHDA infusion significantly decreased the number of cells in the SN at all time points (**$p < 0.001$). Infusion of GDNF did not prevent a loss of TH^+ in SN of 6-OHDA animals at 2 wk (**$p<0.001$), whereas no significant loss of TH^+ was observed in GDNF-treated 6-OHDA animals by 8 wk. 6-OHDA infusion significantly decreased the number of FG+ cells in the SN at 2, 4 and 8 wk and this was greatly attenuated by GDNF (**$p < 0.001$). All values are expressed average number of cells ± S.E.M.

***Effect of GDNF on the 6-OHDA-induced loss of TH positive neurons*:** 6-OHDA caused a 50% loss in TH^+ cells in the SN at 2 wks post-6-OHDA infusion that persisted for at least 8 wks ($p < 0.001$). GDNF

alone had no significant effect on the number of TH^+ cells at any time point ($p > 0.05$), and as in the case of striatal markers of DA terminals, GDNF infusion prior to 6-OHDA did not prevent the loss of TH+ cells in the SN at 2 wks. However, by 8 wks there was no longer any significant difference between the number of TH^+ cells in the SN of animals treated with GDNF + 6-OHDA alone and vehicle-treated animals ($p < 0.001$; Figure 3, previous page).

6-OHDA-induced loss of TH^+ cells in the SN could have been a result of a loss of the TH phenotype rather than actual cell death or degeneration. Therefore, we injected FG into the striatum 1 wk prior to administering 6-OHDA ± GDNF into the same region, and the number of FG^+ cells in the SN, as well as cells co-labeled with TH and FG was then determined. A 57% loss of FG^+ cells was observed at 2 wks in animals given 6-OHDA alone ($p < 0.001$), which was even greater at 4 wks (78%; $p < 0.05$) and 8 wks (83%; $p < 0.001$) (Figure 3), indicating that 6-OHDA caused a gradual but profound loss of the DA cells projecting from SN to striatum.

In summary, GDNF given just prior to 6-OHDA protects the DA neurons from 6-OHDA. However, whereas no significant loss of cells was detected over the period examined (2 – 8 wk), it took 8 weeks for the restoration of the major phenotypic markers of DA neurons. (In our summary of accomplishments last year, we alluded to this work. However, since we were still completing the project no data were presented.)

2. The effects of GDNF may not be fully explainable in terms of the activation of ERK

A major focus of our work is to determine the signaling cascades associated with GDNF-induced protection of DA neurons. We have found that exposure of ***MN9D*** cells to GDNF (10-100 ng/ml for 15 min) results in an increase in ERK1/2 and ERK5 *phosphorylation*, an increase in ERK *activity* as assessed in a kinase activity assay using the transcription factor Elk1, a well-known ERK target, and an increase in *pCREB*. The activation of ERK appeared to participate in the neuroprotective effects of GDNF since U0126, an inhibitor of MEK, eliminated the protective effects of GDNF. We also have examined the effect of forced limb use and of GDNF on activation of ERK1/2 in our ***casted animal model of PD***. Western immunoblotting showed that forced use of one limb increased pERK1/2 by 4-fold in the contralateral striatum at 24 hr, and levels remained high for the 7-day casting period. In the SN, the increase in pERK1/2 was more gradual but reached a comparable level within 7 days. This work is still in progress.

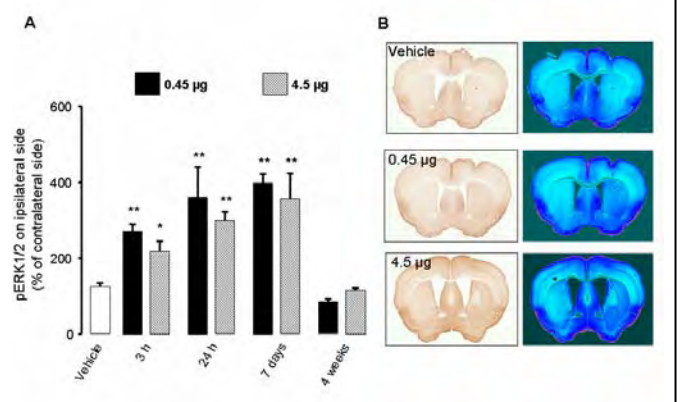

Figure 4: Effect of intrastriatal GDNF on striatal pERK1/2 as a function of time. Animals received vehicle, 0.45 µg GDNF or 4.5 µg GDNF into the striatum. After 3h, 24h, 7 days, or 4 weeks the animals were perfused and brain sections were labeled with anti-pERK1/2. A. The labeled sections were scanned and quantified using Metamorph software. The measured immunoreactivity in the ipsilateral striatum is expressed as a percent of the non-injected contralateral striatum in all animals, including vehicle controls. Data are mean ± SEM. *P<0.05, **P<0.01 vs. vehicle, ANOVA followed by Dunnett's. B. Representative slides of a vehicle injected animal (top), an animal injected with 0.45 µg of GDNF (middle), and an animal injected with 4.5 µg of GDNF (bottom). The slides to the left are unaltered scanned images, whereas the images on the right were modified with Metamorph software to visualize intensity differences.

Effect of GDNF on pERK levels in mouse striatum: In the meantime, we have established a mouse model in which to examine the effects of GDNF in order to take advantage of the availability

of mouse lines in which one or more NTF or NTF receptor is knocked out. In these studies, GDNF was infused into the striatum and striatal pERK1/2 was analyzed by immunohistochemistry at 24 hr. GDNF caused a unilateral dose-dependent increase in the phosphorylation of ERK1/2 in the striatum that was significant at 0.045 µg GDNF and peaked at 0.45 µg, reaching 330% of vehicle control. At 4.5 µg of GDNF, the rise in phosphorylation appeared to decrease slightly from the effect at 0.45 µg. However, this decrease was not significant. Comparable data were obtained via Western blot analysis.

The temporal effects of high and low GDNF on pERK1/2: The infusion of 0.45 µg GDNF caused a time-dependent increase in pERK1/2 in the ipsilateral striatum analyzed by immunohistochemistry that was detectable by 3 hr ($p < 0.01$), reached 420% above control by 7 days but was back to basal levels by 4 weeks. Infusion of 4.5 µg GDNF also caused a time-dependent increase in pERK1/2 that appeared slightly lower than the effect of 0.45 µg GDNF, although the difference was not significant. Seven days after administration of 4.5 µg GDNF there was a small region of downregulation of ERK1/2 phosphorylation around the needle track despite the fact that pERK1/2 was still increased in the remaining ipsilateral striatum (Figure 4 on previous page).

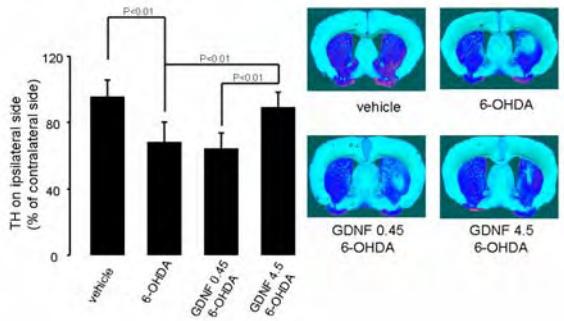

Figure 5: Effect of GDNF on the loss of tyrosine hydroxylase induced by 6-OHDA. Animals received vehicle, 0.45µ or 4.5 µg of GDNF into the striatum. Six hrs later, 6-OHDA (0.5 µg / 0.5µl at 0.1 µl/min) or saline was infused in the striatum at the same site as the GDNF infusion. After 4 weeks the animals were perfused and brain sections were labeled with an antibody to TH. Sections were then scanned and quantified using Metamorph software. The measured immunoreactivity in the ipsilateral striatum is expressed as a percent of the non-injected contralateral striatum in all animals, including vehicle controls. Data are mean ± SEM values.

Effect of high and low dose GDNF on toxicity induced by 6-OHDA: Mice injected with vehicle, 0.45 µg or 4.5 µg GDNF received 6-OHDA (0.5 µg) into the striatum 6 hr later. After 4 weeks, the mice were sacrificed, and the presence of DA terminals in the striatum was analyzed by immunohistochemistry for TH, a marker for these neurons. 6-OHDA by itself caused a large loss of TH immunoreactivity at the site of toxin injection. Injection of 4.5 µg GDNF significantly reduced the 6-OHDA-induced loss of TH immunoreactivity by 75%. In contrast, 0.45 µg GDNF did not have any apparent effect on the 6-OHDA-induced TH loss (Figure 5).

In summary, GDNF protects against the effects of 6-OHDA in the mouse as in the rat. However, contrary to our in vitro results, whereas 0.45 µg GDNF produced a maximal increase in striatal pERK levels, it did not protect against 6-OHDA – a significantly higher concentration of GDNF was required for the latter effect. We will be examining other possible explanations for the cellular basis of GDNF protection, beginning with a possible role for Akt.

4. Long-term proteasome inhibition in PC12 cells elicits protection against 6-OHDA.

Last year we summarized our work on the apparent capacity of DA cells to mount a defense against cellular stress. That work, published in three papers (Cavanaugh et al., 2006; Leak et al., 2006, and Lin et al., in press, 2007), indicated the following: (a) 6-OHDA increased pERK1/2 in MN9D cells, while having little effects on ERK5, (b) blockade of this increased pERK was associated with an increase in the toxic effects of 6-OHDA, and (c) pretreatment with a sub-toxic concentration greatly attenuated the toxic effects of a subsequent higher concentration of 6-OHDA in MN9D cells. This work is continuing along several paths. In one set of studies we are using a 6-OHDA/rat model to explore the assumption that preconditioning also occurs in vivo. We will report on those results next year.

In a second, major set of experiments, we have selected PC12 cells, a line more generally available than MN9D cells and about which considerably more is known. PC12 cells have the added advantage that they do not change with successive passages as much as do MN9D cells and thus seem a better model for longer experimental time frames. In our initial studies, we found that PC12 cells did not take up tritiated DA. Thus, we chose to make use of PC12 cells stably transfected with human DA transporter (kind gift of Dr. Gonzalo Torres at the University of Pittsburgh) to ensure rapid high affinity uptake of 6-OHDA into the cell within a short exposure interval. We first confirmed a high expression of TH and nomifensine-sensitive DA uptake. Given that cellular stress is often chronic, we decided to examine a long-term treatment to test the hypothesis that otherwise healthy cells exposed even to a chronic insult would be protected against subsequent insults. 6-OHDA was not suitable for long-term exposures because it rapidly oxidizes under culture conditions. Thus, we applied the proteasome inhibitor MG-132 chronically to PC12 cells. Cells were grown for 2 weeks in 0.1μM MG-132 and then continuously maintained in the presence of this inhibitor.

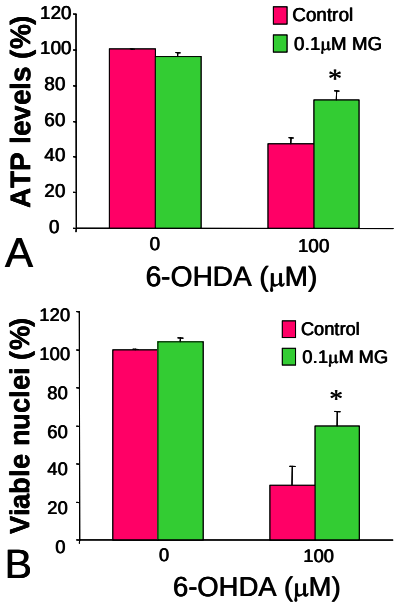

Figure 6: PC12 cells chronically treated with 0 or 0.1 μM MG-132 were exposed to a challenge of 100 μM 6-OHDA. Cells were lysed and assayed for ATP levels (A) or stained with Hoechst reagent (B) 24 hrs after the challenge. * $p < 0.05$ compared to non-MG-132 treated control cells, Bonferroni post-hoc.

We observed that this treatment protected PC12 cells against a toxic concentration of MG-132 (40 μM) as well as against a toxic dose of 6-OHDA (100 μM, Figure 6), as well as the effects of 6-OHDA. As in the case of our previous studies of acute protection of MN9D cells, this phenomenon could not be explained by a reduction in toxin uptake through the DA transporter – ^{3}H-DA uptake was actually slightly increased in the MG-132 pretreated cells. We observed some cell death in the PC12 cells maintained in MG-132; moreover, the cells continue to divide during the period of MG-132 exposure. Thus, we do not use the term "preconditioned" when describing this insult. Nonetheless, we do hypothesize that self-defensive responses were mounted by the remaining cells. We are currently examining the mechanism of this defense. Not surprisingly, there appear to be distinctions between the MN9D and PC12 results.

5. Effect of wildtype and mutant LRRK2

As indicated in our Introduction, PD is likely to involve interactions between environment and genetics. Recently, mutations in LRRK2 have been identified as a major factor in PD. Moreover, LRRK2 contains a kinase domain, raising the possibility that its actions are related in

some way to the NTF-kinase axis that is central to our interests. Thus, we have decided to characterize functional differences between wild-type LRRK2 and Y1699C mutant in response to oxidative stress. The constructs containing human LRRK2 wild-type gene and Y1699C mutant fused with GFP at the C-terminal in the mammalian expression vector pCDNA3.1 were kind gifts from Dr. Matthew Farrer and the Neurogenetics Laboratories, Mayo Clinic, Jacksonville. In our initial characterization, we have selected HEK293 cells as the host for the wild-type LRRK2 and Y1699C mutant and H_2O_2 as the stressor. This allows us to achieve high transient transfection efficiency and a readily reproducible insult in order to begin to examine the initial interaction between LRRK2 and oxidative stress.

Changes in basal cell physiology due to expression of wild-type LRRK2 and Y1699C mutant: First, we examine changes in basal cell viability and basal levels of pERK and pJNK due to expression of wild-type LRRK2 or Y1699C mutant in HEK293 cells. To ensure high transfection efficiency, we monitored the expression of each LRRK2 gene by fluorescence microscopy via their GFP moiety fused to their C-terminal, finding that at least 70% of our cells exhibited the green fluorescence demonstrating the efficacy of this approach. The presence of wild-type LRRK2 and Y1699C mutant in HEK293 cells was also visualized by probing immunoblots with an antibody recognizing GFP. No band was detected in lysate derived from cells transfected with the empty vector (pcDNA3.1). We have also failed to detect endogenous wild-type LRRK2 in HEK293 cells with several commercially available antibodies.

Expression of either wild-type or mutant LRRK2 decreased the pERK level, with the mutant producing a larger decrease than the wild-type when compared to cells transfected with pcDNA3.1. These changes were not associated with alterations in total ERK. Moreover, no visible changes in the expression level of pJNK and total JNK were detected. Expression of wild-type LRRK2 and the Y1699C mutant for 24 hours consistently resulted in a 40% drop in basal cell viability of HEK293 cells. A similar observation has also been reported in studies where wild-type and mutant LRRK2 were expressed in other cell types, suggesting that over-expression of these proteins in mammalian cells could be toxic.

Cells expressing wild-type LRRK2 is more resistant to oxidative stress than cells expressing Y1699C mutant: Next, we examined the interaction of LRRK2 and H_2O_2, using a concentration of the peroxide that is roughly at the EC50 for toxicity (150 μM). At 4 hr after the plasmid transfection process no visible cell death was observed. However, at 18 hr of H_2O_2 treatment, we observed that cells expressing LRRK2 wild-type gene exhibited higher resistance towards H_2O_2 toxicity with 27% cell death as opposed to 50% cell death in cells expressing Y1699C mutant under the same treatment (Figure 7A, next page).

Cells expressing wild-type LRRK2 elicited a significantly higher pERK level in response to H_2O_2 toxicity than cells expressing Y1699C mutant (Figure 7B, next page). No such change was observed for pJNK. Thus the results suggest potential cross-talk between LRRK2 and the ERK pathway. Moreover, the observed higher level of pERK consistently correlated with a lower H_2O_2-induced cell death in cells expressing wild-type LRRK2 as compared to those expressing Y1699C mutant. We intend to perform subsequent experiments to examine any causative relationship between ERK activation and attenuation in cell death.

In summary, it appears that the wild-type form of LRRK2, but not the Y1699C mutant reduces the vulnerability of HEK293 cells to the oxidative stress imposed by H_2O_2. This is associated with an increase in pERK, which also is produced by wild-type gene expression.

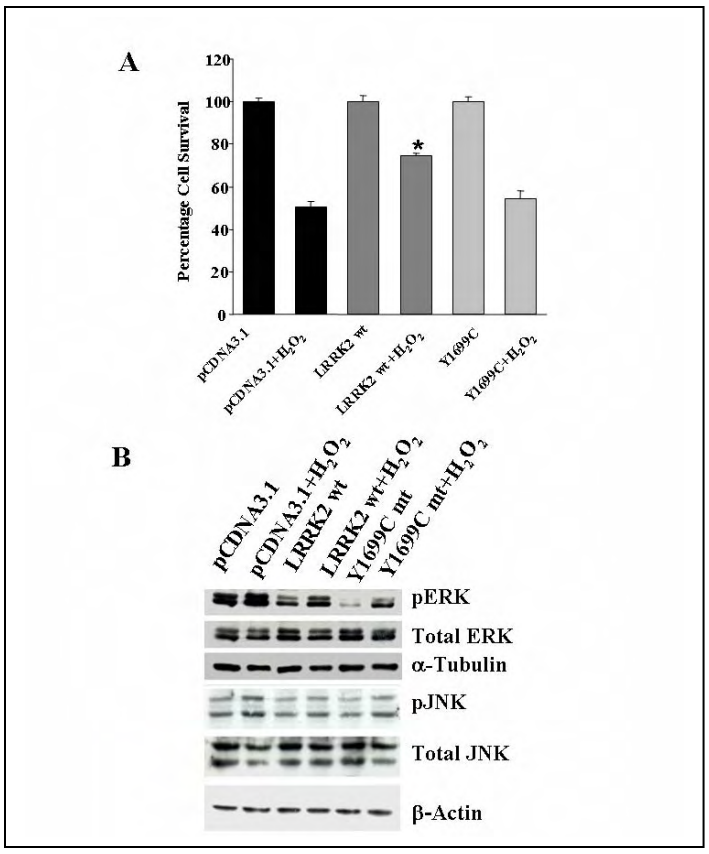

Figure 7: Impact of LRRK2 wild-type gene and Y1699C mutant gene expression on H2O2-induced cell death. (A) Attenuation of H_2O_2-induced cell death for cells expressing LRRK2 wild-type gene, but not in cells expressing Y1699C mutant gene. Percentage cell death induced by H_2O_2 among cells transfected with pCDNA3.1, expressing LRRK2 wild-type or Y1699C mutant gene is normalized against untreated cells transfected with the same vector or expressing the same proteins. Data are means ± SEM, at least 24 readings per data point, from six independent experiments. * $p < 0.05$ versus viability of cells transfected with pCDNA3.1 after 150 μM of H2O2 treatment for 18 hours. (B) Corresponding changes in activated ERK, total ERK, activated JNK, and total JNK in HEK293 cells expressing LRRK2 wild-type gene or Y1699C mutant gene with and without chronic treatment with 150 μM of H_2O_2 for 18 hours. β-Actin was used as loading control.

Key Research Accomplishments:

- GDNF protected DA neurons in both rats and mice against 6-OHDA. However, full restoration of the DA phenotype required 4-8 weeks.
- pERK1/2 was associated with the neuroprotective effects of GDNF in DA cells studied in culture. However, in our animal model, the protective effects of the NTF could not be fully explained by changes in pERK and is likely to involve other signaling cascades.
- Long-term inhibition of proteasomal function increased the resistance of PC12 cells to the toxic effects of proteasome inhibition (MG-132) and oxidative stress (6-OHDA).
- LRRK2 increased resistance of HEK293 cells to oxidative stress (H_2O_2) more effectively that did the Y1699C mutant. This was associated with an increase in pERK1/2 cells produced by the wild-type but not the mutant form of the gene.

Reportable Outcomes:

- GDNF protects DA neurons from 6-OHDA. However, the restoration of a DA phenotype requires several weeks, presumably because the neurons temporarily alter their pattern of gene expression so as to focus on combating the toxic effects of oxidative stress.
- The proteasomal inhibition increases the resilience of PC12 cells to subsequent cellular stress.
- The LRRK2 gene appears to reduce the vulnerability of HEK293 cells to oxidative stress. This effect may be related in part to its ability to activate ERK1/2. The protein product of the LRRK2 mutant Y1699C does not have this neuroprotective property not does it have a significant activating effect on ERK.

Conclusions and Plans for Coming Year:

Exercise continues to be a viable approach to the treatment of PD. This is made clear by our own work and that of several other groups studying both animal models and humans. Understanding the parameters of protective exercise will be critical to proscribing an effective exercise regimen. Moreover, understanding the mechanism of action of exercise should provide insights into the development of drug therapies. Much of the last year has been spent exploring the effects of GDNF and 6-OHDA, as well as the role phospho-kinases play in their actions vis-à-vis cellular viability. Much of this work will continue. Specifically, we will explore the following:

1. What are the events that occur during the first few weeks after intracerebral administration of GDNF immediately prior to 6-OHDA? Are there behavioral deficits, what are the extracellular DA levels, and what underlies the delay of several weeks prior to full restoration of the phenotype?

2. What are the relative role of pERK and pAkt in the neuroprotective effects of GDNF during exposure to oxidative stress?

3. What intracellular events are associated with defensive response of DA cells to oxidative stress?

4. What is the mechanism of action of the neuroprotective effects of long-term proteasome inhibition?

5. How does LRRK2 provide protection against oxidative stress in HEK293 cells and its relevance to DA neurons?

We will also be returning to the study of exercise. Our initial studies on exercise were carried out in collaboration with the group of Dr. Tim Schallert (University of Texas, Austin) and utilized unilateral forelimb casting to produce increased motor activity in the contralateral limb. However, neither Dr. Schallert nor we have been very successful recently in obtaining robust effects with this model. Thus, we have begun to explore two other models, treadmill running and wheel running. These studies will use both rats and mice and both 6-OHDA and MPTP. More specifically we will ask:

6. What is the relation between the amount of running and protection against the behavioral effects of 6-OHDA and MPTP?

7. Is behavioral protection associated with protection of DA cells, terminals?

8. How does stress influence exercise-induced neuroprotection?

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